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A STUDY OF THE HEMAGGLUTININ OF CLOSTRIDIUM BOTULINUM

Prepared for:

ARMY RESEARCH OFFICE
3045 COLUMBIA PIKE
ARLINGTON, VIRGINIA 22204
ATTN: LIFE SCIENCES DIVISION

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By: LLOYD K. MOSS

SRI Project BU-5280

Approved: W. A. SKINNER, EXECUTIVE DIRECTOR
LIFE SCIENCES RESEARCH

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I INTRODUCTION

Botulinum toxin is the most potent natural poison known to man. Considerable information is available concerning the microbiology of five of the six types of Clostridium botulinum, the production of type-specific toxins, and the isolation and characterization of these toxins. From crude to highly purified material, the toxins are associated with hemagglutinins. Only under extraordinary conditions is it possible to separate these components.

Research emphasis in the past has been on the toxin. At present, the greatest gap in knowledge of the biochemistry of the botulinal toxin complex is in the physical chemical interrelationships between the neurotoxin and the hemagglutinin. A similar "lock and key" relationship undoubtedly exists between a different set of reactive groups of the hemagglutinin and the surface of erythrocytes.

At the outset, the objectives of this project were:

(1) To attempt separation of the hemagglutinin from the neurotoxin, and to purify and isolate it;

(2) To characterize the isolated hemagglutinin by chemical, biophysical, and immunochemical methods; and

(3) To investigate the physical chemical interrelationships between the neurotoxin and the hemagglutinin.

As the research progressed, it became clearer that characterization of the hemagglutinin was important to the detection of the complex. Consequently, detection by hemagglutination methods was emphasized over animal toxicity studies.

The studies described pertain only to type A botulinum toxin.

II SUMMARY AND CONCLUSIONS

1. The hemagglutinin of type A toxin behave in gel filtration chromatography like a high molecular weight substance, separating into two peaks of hemagglutination activity that may be related as monomer and dimer.

2. There was no relationship between the ultraviolet absorption of hemagglutinin solutions and the hemagglutination activity.

3. Amino acid analyses were performed on partially purified mixtures of neurotoxin and hemagglutinin, a high molecular weight component free of hemagglutinin, a related high molecular weight component with the greatest hemagglutination activity of the chromatogram, and crystalline toxin. There were close similarities in amino acid composition. The hemagglutinin probably contains little tryptophan because of the independence from ultraviolet absorbance. The absence of methionine in the initial high molecular weight peak suggests that it is maybe a nontoxic impurity.

4. The concentration of hemagglutinin in the partially purified toxin-hemagglutinin complex is estimated at 1% by weight.

5. The hemagglutinin probably is a protein, in agreement with other hemagglutinins, but this fact must be firmly established.

6. It would be unwise to depend on identification of botulinum toxin by its property of hemagglutination, because this activity can be easily removed without seriously diminishing the toxicity.

7. Minimum detection of hemagglutinin by conventional settling patterns with chicken RBCs occurs at 6,000 mouse MLD_{50} ml or about 0.2 μg toxin ml based on the neurotoxin-hemagglutinin complex. Use of the Coulter Counter may increase the sensitivity 100-fold.

III BACKGROUND

In 1959, Lamanna summarized what was known about the toxin produced by Cl. botulinum.[1] He also listed the problems that remained to be solved concerning this neurotoxin. The following discussion is restricted to type A toxin and its hemagglutinin.

In general, botulinum toxin is a globulin-like protein[2,3] that has been purified to an essentially homogeneous, crystalline material. Its molecular weight, approximately 900,000,[4,5] can represent the sum of hemagglutinating and toxic sub-units combined in a protein complex.[6,7] The isoelectric point of the complex is pH 5.5.[2] It is readily precipitated by ammonium sulfate and other protein precipitants.[2,3,8] The amino acid composition[9,10] gives no clues to the potent neurotoxic activity or to its ability to agglutinate red blood cells and to become adsorbed to other cells and tissues.

The solubility of the purified toxin-agglutinin complex in dilute aqueous salt solutions is limited to less than 1%. A solution of that concentration would have the lethal power of 320,000,000 LD_{50} units/ml when injected intraperitoneally into white mice.[11] The toxicity in man has not been defined adequately.

Lamanna[6] was the first to report that RBC's (red blood cells) agglutinate in the presence of botulinum toxin. The hemagglutination could be followed either microscopically or by macroscopic methods in use for observation of virus hemagglutination, e.g., Hirst[12,13] and Salk.[14] Botulinum toxin not only aggultinates RBCs, but precipitates a broad electrophoretic range of serum proteins and other proteins, such as pepsin.[15] Lamanna and Doak[15] observed, however, that the toxin did not precipitate solutions of lactoglobulin; gelatin is another exceptional protein. Although heparin is not a protein, Hildebrand, Lamanna and Heckly[16] presented evidence that it precipitated toxin from salt solution. Heparin did not interfere with toxicity in vivo, hence it may have been a selective agent for the hemagglutinin.

In three papers[7,17,18] on various aspects of botulinal hemagglutination, Lamanna and Lowenthal reported that commercially available type B antitoxin is capable of neutralizing the hemagglutinating activity, but not the toxicity, of type A toxin preparations. By exposing RBCs to botulinum toxin at low temperature and then eluting them at 37°C, a relatively pure hemagglutinin was obtained and studied by gel diffusion techniques.[7] In subsequent research, the hemagglutination reaction was pursued to develop a reliable quantitative method for assaying the toxin. Cell concentration, time of incubation, temperature of incubation, salt concentration, and pH all had an effect on the hemagglutination titer and

made it difficult to equate with toxicity titer. Some preliminary results of heat, formalin, and acidity on toxicity and hemagglutination titers were obtained.[17]

Lowenthal and Lamanna[18] also observed that the dissociation of the toxin and hemagglutinin is directly related to the pH, the optimum pH for hemagglutination being 7.7. The rate of adsorption of the hemagglutinin is almost instantaneous at low temperatures. They concluded that the mechanism for botulinal hemagglutinin-RBC attachment is apparently similar to that proposed for virus-host cell attachment, namely, an electrostatic attraction. However, pre-absorbed influenza virus did not inhibit botulinal hemagglutination.[18]

Lamanna and Aragon[19] briefly described preliminary evidence of the nature of the RBC receptor for the botulinal agglutinin. Both formalin and tannic acid treatment of RBCs decreased uptake of hemagglutinin. Treatment of RBCs with proteolytic enzymes, such as papain and trypsin, produced a similar loss of hemagglutinin adsorption capacity. This was considered evidence in favor of the protein nature of the botulinum hemagglutinin receptor site on the RBC.[19]

Studies by Meyer and Lamanna[20] centered on the effect of proteolytic enzymes on the neurotoxin and hemagglutinin. Denatured egg albumin was used for the selective adsorption elution of the hemagglutinin. Enzymes investigated included trypsin, chymotrypsin, pepsin, botulinal amino-peptidase, papain, ficin, and Bacillus subtilis glutamyl transferase. The activity of the hemagglutinin was not affected, although these enzymes, with the exception of pepsin and papain, did destroy toxic activity. Halliwell[21] reported no toxicity in certain trichloroacetic acid soluble fractions after proteolysis of pure toxin. Trypsin caused extensive inactivation; pepsin was only slightly active and papain was ineffective. In contrast, Coleman[22] observed inactivation of crystalline toxin by pepsin and chymotrypsin, but the toxin was resistant to tryptic action.

In research on the sedimentation behavior and hemagglutination activity of complexes dissociated at neutral pH, Wagman and Bateman[23-25] obtained a slowly sedimenting fraction formed at pH 7.5 with an estimated molecular weight of 71,000 and with no hemagglutinating activity. Wagman described a procedure for isolation of the low molecular weight form of botulinum toxin.[23] Another fraction at pH 7.5 (μ 0.13) lost its hemagglutinating capacity; however, when the pH was adjusted to 3.8, agglutination was restored.[23] Wagman[25] credited M. S. Davis and P. A. McCaffrey with showing conclusively that the hemagglutinin is removed from toxin solutions when RBCs are agglutinated, without bringing about any measurable change in the sedimentation diagram of the toxin. In addition, the agglutinin particles were concluded to be very small nontoxic units, because they could be removed from the toxin without measureable loss of total protein or toxicity.[23]

Evidence presented by Wagman[26] confirmed the alkaline dissociation of the toxin in Tris buffer at pH 9.2. On the other hand, irreversible

dissociation was indicated by a lack of reaggregation when the pH was adjusted to 3.8. The toxin-hemagglutinin complex was highly resistant to peptic digestion in its natural conformation with a molecular weight of 900,000. In contrast, dissociated toxin with a lower sedimentation constant was extensively degraded by pepsin but retained considerable toxicity. No information on the behavior of the hemagglutinin or hemagglutinating activity of the treated solutions was presented.

The biosynthesis of the hemagglutinin is coincident with toxin production. In the purification procedure employed to date,[27] hemagglutinin accompanies the toxin even in the crystalline product.[7,3]

The toxin-hemagglutinin complex has been subjected to a variety of chemical agents. It is sensitive to oxidative denaturation by iodine[28,29] and ferric ion[30] and is photochemically inactivated in the presence of methylene blue.[31] In their work on fluorescence changes of botulinum toxin solutions, Boroff and Fitzgerald[30] studied the effect of dilute solutions (0.01 to 0.001 M) of several metal ions. Only ferric ion showed any effect on fluorescence or toxicity; it depressed toxicity at higher concentrations.

More recently, Boroff and co-workers[32] pursued the hypothesis that fluorescence, tryptophan, and toxicity are closely linked. Schantz, Spero, and co-workers studied the reaction of botulinum toxin with ketene,[33] nitrous acid,[34] and alkali.[35] They reported that urea irreversibly destroys the toxicity of the toxin without changing the fluorescence. It is highly probable that cyanate in the urea is the toxoiding agent.[36,37] Brazis et al.[38] described the effectiveness of halogens or halogen compounds in detoxifying botulinal toxins. Repeated subculturing of Cl. botulinum results in a gradual loss of toxicity with eventual complete suppression of toxin production, according to Boroff.[39]

In each of these studies, the salient criterion of reaction was change of toxicity. Hemagglutination was seldom studied, much less emphasized.

IV EXPERIMENTAL

A. Preparation of Toxin Solutions

1. Stock Solution

A moderate supply of partially purified, spray-dried Type A toxin remained from previous studies (Contract DA 18-108-AMC-176 [A]). A stock solution was prepared by suspending 1 g of crude toxin in 100 ml of 0.05 M phosphate buffer (pH 7.3). A magnetic stirrer was used for 10 min to aid solution. The suspension was centrifuged for 10 min at 1000 x G and the faintly turbid, amber-colored supernatant solution was removed with a syringe and needle and transferred to sterile serum bottles for storage at 4°C. The sediment was resuspended in another 100 ml of the same phosphate buffer and again stirred, centrifuged, and separated to obtain a second extract. The sediment in the centrifuge tubes was discarded.

2. Solution of Crystalline Toxin

A 50-mg sample of crystalline Type A toxin was received from Dr. Edward J. Schantz, U.S. Army Biological Laboratories, in September, 1963. The sample, in the form of a solution of toxin in 17 ml of 0.05 M sodium acetate buffer (pH 3.8) was stored at 4°C until used. Portions of the solution were removed for gel filtration chromatography and for hemagglutination studies.

3. Up-grading of Crude Toxin

The preparation of high purity toxin was attempted by a slight modification of the procedure of Duff and co-workers.[27] A 1-g sample of dry chemical agent was slurried in 50 ml of 0.075 M calcium chloride solution, adjusted to pH 6.6 by addition of 2 N sodium hydroxide, and stirred with a magnetic bar for 30 min. The insoluble materials were removed by centrifugation at 1000 x G for 10 min in 50-ml Autoclear centrifuge tubes. (Note: Although the supernatant solution was amber-colored, it was perfectly clear, in contrast to turbid solutions obtained with phosphate buffers. It was transferred to a beaker with the aid of a syringe and needle. The pellet of almost black "mud" was discarded. The calcium chloride extract was adjusted to pH 3.7 with 1 N hydrochloric acid, allowed to settle at 4°C overnight, and centrifuged for 10 min at 1000 x G as a cold suspension. The supernatant solution was removed with a syringe and needle. The white sediment of acid mud was dissolved in about 6 ml of 0.03 M phosphate buffer (pH 6.8), and gave a clear solution. After 2.5 ml of 50% ethanol was added, bringing the solution to 17% ethanol, the turbid solution was kept at 4°C for 24 hr. After centrifugation in the cold for 10 min at 1000 x G, the clear supernatant solution was removed. The alcohol-precipitated sediment

was dissolved in about 5 ml of 0.03 M phosphate buffer (pH 6.8) and clarified by filtration through a Gelman Cytosieve filter. A total of 3.0 ml 4 M ammonium sulfate solution was added dropwise to the filtrate. The resulting suspension of crystalline toxin was stored at 4°C in the ammonium sulfate solution.

B. Mouse Assays

The buffer used for dilution of toxin contained 0.2% gelatin in 1% dibasic sodium phosphate and was adjusted to pH 6.8 with hydrochloric acid.[33] It was sterilized by heating in an autoclave for 30 min. Concentrated toxin solutions were diluted in steps of 1:100 to a concentration containing at least 10 lethal doses per ml. These solutions were serially diluted two-fold to a concentration that was sublethal to all animals in the group. Groups of five white, random-bred Swiss-Webster mice (Simonsen Laboratories, Gilroy, California) weighing 20 to 22 g, were injected intraperitoneally with 0.1 ml of the dilutions of toxin and observed for four days. The number of dead mice was recorded daily, and dead animals were removed from the cages.

The number of mouse intraperitoneal lethal doses per milliliter ($MIPLD_{50}$/ml or, more commonly, MU/ml) was determined by the graphic method of Weiss,[29] plotting on K&E probability paper (#358-22) the number of animals dead in each group (dilution) at 96 hr. Alternatively, the tabular method of Weil[40] was used. The 95%-confidence interval was estimated by the latter procedure. In brief, the toxin concentration in the original solution was equivalent to the reciprocal of the dilution producing the $MIPLD_{50}$.

C. Direct Hemagglutination Titration

1. Hemagglutination Titration Procedure

In a hemagglutination (HA) titration experiment, highly active toxin solutions (10×10^6 MU/ml) were diluted to a concentration of about 100,000 MU/ml using Bacto-Hemagglutination buffer of Wheeler and co-workers[41] as the diluent. A 0.50-ml sample of the diluted stock solution, or effluent from Sephadex chromatography, was transferred with a 1-ml Tuberculin syringe with a 24-gauge needle into the first well of a Perspex plastic HA tray[42] (Instrumentation Associates, Inc., 16 West 60th Street, New York 23, N.Y.). Another 0.50-ml sample was serially diluted (two-fold) in the same HA buffer in subsequent wells of the HA tray, with precautions to obtain adequate mixing at each concentration. To the 0.5 ml of solution in each well was added 0.10 ml of 1.25% suspension of fresh, washed red blood cells (RBCs) of the particular species being tested. The RBCs were delivered from a 1-ml Tuberculin syringe with a 20-gauge needle. The suspension and contents of each well were mixed with a small disposable pipette, starting from the well of highest toxin dilution and working backward to the well of highest toxin concentration. A 0.10-ml aliquot of the cell suspension placed

in 0.050 ml of the HA buffer served as a control, giving a negative settling pattern unless the RBCs spontaneously agglutinated. A clear sheet of Lucite was placed over the tray, and the settling pattern was observed after a period of from 30 min to several hours.

After the settling patterns were recorded, the plastic trays and toxic contents were decontaminated by flooding with dilute (10%) Chlorox solution.[43] Later, the trays were rinsed with tap water, washed thoroughly in warm detergent, rinsed in tap water and distilled water, and set aside to drain dry. Trays were numbered to aid identification.

Decontaminated and rinsed trays occasionally gave unreliable and irregular HA patterns. The most common unpredictable problem was false positive patterns, attributed to surface effects in the wells. Reliable results were assured only if the tray was set up with HA buffer and if each well gave a definite negative control pattern. When the surface in the wells of a tray became sufficiently abraded by needle wear, it failed to give a definite negative pattern and was discarded.

2. Preparation of Erythrocytes

The RBCs of a number of species were obtained from the Colorado Serum Company on a weekly basis as a solution of 50% blood in a modified Alsevers solution. The blood samples, as received, were stored at 4°C until the RBCs were washed for use.

The blood was centrifuged at 350 x G (1500 rpm) in graduated centrifuge tubes of heavy-walled Pyrex glass. The red cells were washed four times in cold HA buffer and stored at 4°C as a 50% suspension and subsequently diluted with HA buffer to a 1.25% suspension. A 0.10-ml sample was added to each well for the HA test. In the case of chicken RBCs, this was about 1.0×10^7 cells/well.

3. Coulter Counter Studies

A Coulter Electronic Particle Counter (Model B), equipped with a 100-μ aperture and a 500-μl manometer, was used with the lower threshhold setting of 6.0 and the upper setting unlimited. Preliminary trials indicated that 0.1 ml of 1.25% suspension of chicken RBCs in 0.5 ml of HA buffer required a dilution of 1:100 to obtain a suspension appropriate for counting. Thus, 0.1 ml of the typical HA titration suspension was diluted to 10.0 ml with HA buffer in a vial, thoroughly mixed, and counted. A plant lectin solution (Bacto-Phytohemagglutinin-P, dissolved and diluted in HA buffer) was used for the hemagglutination studies with the Coulter Counter. The background count of the HA buffer was less than 2% of the RBC count.

D. Gel Filtration Chromatography

A quantity of dry Sephadex G-200, varying from 1.3 to 5 g according to the size of the column, was allowed to swell for at least 72 hr in

500 ml of HA buffer. The slowly sedimenting fines were removed by aspiration during repeated settling steps, and the more dense gel was packed by gravity settling in HA buffer in Sephadex Laboratory Columns. The packed column was washed and developed exclusively with HA buffer (pH 7.2).

The dimensions of the initial 2-g column were 2.5 cm x 11.0 cm; total volume, 55 ml; void volume, 17 ml; ratio of height/diameter was 4.4. A column prepared with 4 g of Sephadex G-200 had the following dimensions: 2.5 cm x 22.8 cm; total volume, 112 ml; void volume, 34 ml; ratio of height/diameter, 9.1. Another column was prepared using the narrow 90-cm column, packed with approximately 5 g of Sephadex G-200; successful use of this column was prevented by precipitation of the proteins in the loading solution at the top of the gel bed. Later experiments were performed on a narrow 30-cm column. The dimensions of this column, packed with 1.3 g of Sephadex G-200, were: 1.5 cm x 26 cm; total volume, 46 ml; void volume, 13.5 ml; ratio of height/diameter, 17.3.

Performance of the columns and the determination of void volume were checked with a dilute solution of Blue Dextran 2000 before use with protein solutions. Samples loaded on the columns were clear, amber-colored supernatant solutions of toxin (about 1 mg total protein/ml) in HA buffer. The flow rate of the developing buffer was about 45 ml/hr for 2.5-cm I.D. columns and half that rate for 1.5-cm I.D. columns. Effluent was monitored by a Vanguard Automatic Ultraviolet Analyzer (Model 105G-OD) with the following settings: wavelength, 280 mμ; slit, 0.400 mm; scale range, 6.0 to 9.0. Eluting buffer was circulated through the reference cell. The graphic pattern was recorded at a chart speed of 3 in/hr.

Effluent was collected in 4-ml portions with a Vanguard volumetric fraction collector and was transferred to a refrigerator at 4°C for temporary storage at the end of a chromatographic run.

E. Absorption of Hemagglutinin by RBCs

Approximately 1.5 ml of packed erythrocytes from either sheep or ox, harvested and thoroughly washed from suspensions (described under Section C2), were combined with 4 ml of stock toxin solution (described under Section A1) and mixed thoroughly. After 30 min at room temperature, the cells were sedimented at 350 x G (1500 rpm) and the supernatant solution was carefully transferred with a disposable micropipet. A portion of the absorbed solution was compared in a HA titration test against unabsorbed toxin solution, using chicken RBCs as the indicator. Most of the supernatant solution however, was loaded on a Sephadex G-200 column for gel filtration chromatography.

F. Immunochemical Diffusion Studies

Immuno-Plate immunodiffusion plates for use by the double diffusion (Ouchterlony) technique, were obtained from Hyland Laboratories,

Los Angeles, California. The 1 x 3 inch cavity contained 4 ml of agar gel of the following composition: Difco special Noble agar 2%; glycine, 7.5%; sodium chloride, 1%; sodium azide, 0.1%; pH was 7.0 to 7.2. Pattern A was used, and wells had a capacity of 5 µl. Solutions of the antigen (crude stock solution or Type A toxoid solution) were placed in the peripheral wells, and Lederle bivalent botulism antitoxin (Types A and B, equine origin, globulin-modified) was placed in the center wells. The diffusion was performed at room temperature.

G. Amino Acid Analyses

Acid hydrolysates of protein samples (either stock toxin solutions or Sephadex chromatography fractions) were prepared by placing the solution in small Pyrex glass ampules that were then autoclaved for 30 min before being taken from the special toxin laboratory. Hydrochloric acid (5 ml, 6 N, analytical grade acid) was added. The ampule was cooled to -78°C, evacuated by a vacuum pump, and sealed. The ampule was heated for 22 hr at 110°C in an oven (±1°C). After hydrolysis, the ampule was removed from the oven, cooled to room temperature, carefully opened, and the contents quantitatively transferred to a round-bottom flask. Excess HCl was removed by spin evaporation at reduced pressure. The dry residue was dissolved in 2 or 3 ml of sample diluting buffer and a portion of it was loaded onto columns of the analyzer.

Amino acids were determined by ion exchange chromatography in a Beckman/Spinco automatic amino acid analyzer (Model 120),[44,45] using a long path cuvette and accelerated conditions [46,47] applicable to physiological fluids. The elution characteristics of the instrument were determined by analyzing a Type 1 amino acid calibration mixture. Individual constants for each amino acid were used to calculate the quantity of each amino acid indicated on the chromatogram.

H. Health Precautions and Safety Practices

The hazards inherent in experimental studies with botulinum toxin require constant awareness of safety precautions. In general, techniques applicable to the safe handling of the parent bacterial organism were employed, such as those described by Phillips in Technical Study 35[48] and by Chatigny.[49] We found helpful the Toxin Laboratory Safety Manual, prepared by the staff at A. D. Little, Inc.

As a first precaution, all personnel--scientists, technicians, animal handlers, and the janitor--working in the laboratory where the research was conducted were immunized with pentavalent toxoid (Linox 257), an aluminum phosphate-adsorbed product prepared under contract by Parke, Davis and Co., Detroit, Michigan, and provided to us by Dr. Paul J. Kadull, U.S. Army Biological Laboratories. A vial of Lederle Bivalent Botulism Antitoxin (Types A and B, equine origin, globulin-modified; 10,000 units of each type) was kept in the refrigerator in the lab.

All research operations on the active toxin solutions, including preparation and dilution of toxin solutions, hemagglutination studies, centrifugation, Sephadex chromatography, and mouse assays, were performed in the special toxin laboratory. Admission to the lab was limited to immunized personnel. All maintenance tasks in the lab were performed by project staff. A conspicuous sign on the door indicated that hazardous research was being conducted within. The lab was adequately equipped with hoods and absolute filters. Major equipment included a preparative ultracentrifuge, a large autoclave, the automatic fraction collector and absorbance monitor, as well as facilities for mouse assays.

All laboratory manipulations of active toxin, regardless of its form or concentration, was performed by personnel wearing disposable surgical gloves, which were discarded after use. In addition, full-length surgical gowns were worn over normal attire. The gowns were autoclaved after each use, then laundered.

When toxin concentrations exceeded 10^6 MU/ml, regardless of the volume of solution, the investigator was assisted by another staff member who served as a safety man, observing for spills and assisting when needed. A "panic" switch, conspicuously located on a treadle in the laboratory, was used to sound an alarm in an adjacent laboratory if additional assistance was needed within the toxin lab.

After a syringe and needle were used for an HA titration or mouse assay, they were disassembled and immersed in a pipette tray containing 10% Chlorox solution. The plastic HA trays were decontaminated by flooding with 10% Chlorox solution; after 10 min, they were washed in a normal manner. All equipment, bench surfaces, and floors were sponged with dilute Chlorox solution. Other materials were decontaminated by heating in the autoclave.

V RESULTS AND DISCUSSION

A. Characterization of Toxin Solutions

When concentrated stock solutions of partially purified toxin were titrated for hemagglutinin activity at 4°C, the endpoint for chicken RBCs occurred at dilutions of 1:6000 and 1:750 for the first and second supernatant solutions, respectively. Previously, the endpoint for fresh chicken cells at 4°C was about 2200 mouse units (MU)/ml, compared to 9000 MU/ml at room temperature. Accordingly, the first stock solution was presumed to contain approximately 13.5 x 10^6 MU/ml (0.42 mg toxin/ml). The second stock solution extract was about one-eighth as concentrated, using hemagglutination (HA) as the criterion.

Subsequent titrations with chicken RBCs gave endpoints at 1:3200 dilution when the chicken cells were allowed to settle at room temperature. Similar HA titration studies with a sample of crystalline toxin, provided in acetate buffer (pH 3.8) by Dr. E. J. Schantz, U.S. Army Biological Laboratories, gave dilution endpoints at 1:16,000 with chicken cells. The same aliquot, 0.1 ml of 1.25% suspension, of turkey cells was twice as sensitive; the dilution endpoint was 1:32,000. We previously described the variation of HA endpoint with species. Horse RBCs were relatively insensitive to the type A hemagglutinin. Chicken cells were relatively four times as sensitive as sheep cells, which were four times as sensitive as ox cells.

When the stock toxin solution was bioassayed in white mice, it contained 8.0 x 10^6 MU/ml. Approximately one month after the initial mouse assay experiment, we repeated the bioassay with the same solution, which had been stored at 4°C. The resultant potency of 11 x 10^6 MU/ml was considered significantly different. It is doubtful that the enhancement was produced by enzyme action upon the gelatin of the solution. Certainly, it was much less than the 100- to 1000-fold potentiation of activity described by Schantz.[50] Our results emphasize the stability of the toxin solutions. The hemagglutinin activity was also maintained in these solutions.

We had wanted to determine the nitrogen or protein content of this stock solution by direct analysis, but the procedures available to us were extravagant in terms of the sample. For example, the sample requirement for 1 mg of nitrogen is equivalent to approximately 6 mg protein, or more than 180 x 10^6 MU of toxin. The problem was easily and effectively solved by hydrolyzing a small aliquot of the stock solution and determining the amino acid composition. The residual molecular weights for the respective amino acids were used to convert results from micromoles to micrograms. Thus, we found that the stock solution contained 1.09 mg protein/ml. Table I shows the results of this analysis (Col. II) and subsequent analyses, to be discussed.

Table I

AMINO ACID COMPOSITION OF HYDROLYSATES OF PROTEIN SOLUTIONS
(Mole-%)

I	II	III	IV	V	VI	VII
Amino Acid or Compound	Partially Purified Toxin	Peak I F/4-F/5	Trough F/7	Ox-Absorbed Peak I F/5-F/6	Crystalline Toxin	Crystalline Toxin (microbiol.)*
Aspartic Acid	14.63	14.95	16.0	11.23	20.43	17.66
Threonine	5.67	6.93	6.7	6.58	6.79	8.27
Serine	6.62	7.88	8.1	12.22	7.04	4.82
Proline		Trace	2.9		3.11	2.61
Glutamic Acid	11.87	10.73	9.4	8.44	9.51	12.29
Glycine	10.33	9.65	7.5	12.11	4.34	2.14
Alanine	8.27	7.34	7.4	11.82	3.95	5.08
Valine	6.25	4.76	5.3	2.33	4.90	5.23
Half Cystine						0.77
Methionine		Trace	Trace		1.03	0.82
Isoleucine	8.43	5.98	8.0	5.82	8.73	10.57
Leucine	8.90	7.47	8.6	8.44	8.93	9.13
Tyrosine	3.13	3.13	4.7	1.92	6.41	8.66
Phenylalanine	3.66	4.48	4.8	4.37	4.66	0.82
Ornithine		3.80	0.8		0.05	
Lysine	7.95	6.79	5.5	7.74	6.43	6.15
Histidine	1.27	2.85	1.0	2.62	0.75	0.77
Tryptophan	ND	ND	ND	ND	ND	1.05
Arginine	3.02	3.23	3.3	2.91	2.94	3.08

ND - Not Determined

*Calculated from the results of Buehler, Schantz, and Lamanna.[10]

Some additional conclusions may be drawn from the data. Schantz[50] described the lethal potency of 1 mg of crystalline toxin as equivalent to 38 x 10^6 MU, which we used as a toxicity standard. Consequently, the stock solution, prepared from partially purified toxin, contained 0.21 mg toxin protein/ml. We were disappointed to find that the toxin protein represented only 19% of the soluble protein in the stock solution, meaning that there was 81% inert protein. Another disappointing fact was that our stock solution was intentionally prepared as a 1% solution (suspension). Obviously, only 11% of the solids was soluble, and the net soluble toxin was only 2.1%.

The amino acid analysis also gave additional information. The results were compared with the data (Table I, Col. VII) reported by Buehler, Schantz, and Lamanna.[10] Under the assumption that 19% toxin protein should significantly contribute amino acids from its hydrolysate, we would have expected to see proline, cystine, and methionine. There was only a trace of proline and cysteic acid; no cystine or methionine were present. Also, there was a slightly greater mole-% of phenylalanine than of tyrosine. The literature reported a 10-fold excess of tryosine over phenylalanine.[51] We did not determine tryptophan.

We would like to emphasize that these data were obtained with 20 µg of equivalent protein, and recommend the amino acid analysis method as superior to the usual nitrogen determinations for the chemical analysis of botulinum toxin solutions. Additional studies involving amino acid analyses are described in the next section.

An alternative to the earlier methods of Abrams[2] and Lamanna[3] for toxin production of highest purity was described by Duff and co-workers.[27] We followed this procedure starting with the solubilization of the partially purified toxin in the neutral calcium chloride buffer. The most striking difference we observed with the use of phosphate buffers was the great clarity of the solution at this step. The procedure was followed all the way to the crystallization in ammonium sulfate solution, in which state the produce presently exists. Characterization of the crystalline toxin was not pursued. The use of Sephadex chromatography (described below) may be beneficial, increasing yields and toxin activity.

Although Duff et al.[27] reported considerable losses in the purification procedure, they showed that calcium ions clarified neutral solutions and increased the solubility of the toxin under acid conditions, where it is usually recovered by precipitation. In addition, solutions of crystalline toxin containing at least 3 mg/ml were prepared in 0.05 M sodium acetate buffer, pH 3.8. We surmise that calcium acetate buffers should be good solvents for the toxin.

We examined the effect of calcium ion on HA of chicken RBCs. The neutral calcium acetate buffer was serially diluted in the usual Bacto-HA buffer. There was nonspecific agglutination at low dilutions, but above a dilution of 1:50, the pattern was negative. Consequently, since

the stock toxin solution has a dilution endpoint of approximately 1:4000 with the chicken RBCs, it should be possible to determine the effect of calcium ion on the solubility of the toxin, using hemagglutination as the criterion. Of course, total protein and mouse assays would be added as criteria of solubility.

B. Gel Filtration Chromatography

A 5-ml sample of stock solution of type A toxin (40×10^6 MU or 5.5 mg total protein) was loaded on a Sephadex G-200 (2.5 cm x 22.8 cm) column The first components (Peak I) eluted at about 35 ml (the void volume of the column). Thus, they were excluded from the dextran gel and behaved identically with the reference material, Blue Dextran 2000 (avg mol wt 2×10^6) used to calibrate the column. After another 12 ml of effluent, the optical absorbance decreased to a minimum value of about 30% of the maximum at Peak I. Then it gradually increased to the maximum absorbance value of Peak II at an estimated elution volume of 120 ml before returning to baseline at 180 ml of effluent. The curves of the two peaks were smooth and regular, indicating a relatively homogenous distribution of components. Peak II contained more than 90% of the UV-absorbing components on the chromatogram. The pattern resembled that shown in Fig. 1 for a 1.3-g column.

Fraction 11 of Peak I and Fraction 21 of Peak II were arbitrarily selective for HA tests; both gave positive HA patterns at dilutions up to 1:512. Later, the endpoint for Fraction 11 was determined as one-fourth the usual titer of the stock solution. It appears that the HA titer/unit protein increased as some of the components were removed, leaving the hemagglutinin with less opportunity for protein-protein interaction.

We anticipated dissociation of toxin into low molecular weight components but there were no clues to the behavior of the hemagglutinin. To examine the possibility that the high molecular weight peak was slowly undergoing dissociation, Fraction 12 representing 4 ml of Peak I was chromatographed several days later on the same later on the same 4-g column of Sephadex G-200. Since the conditions were essentially the same, only a component equivalent to Peak I appeared on the chromatogram. The column development was stopped at the end of the day without evidence of Peak II. However, when development was continued the next morning, a small absorbance peak occurred. Since the distribution of UV absorbance was 81% and 19% in the two peaks, respectively, either the second peak was an artifact of a small amount of material trapped in the UV monitor, or it was actually Peak II. Another explanation could be that some of the high molecular weight material of Peak I underwent dissociation to lower molecular weight material of Peak II.

A similar chromatogram was run starting with 5 ml of Fraction 22 from the original chromatogram, representing a part of Peak II. This time the chromatogram showed a small peak typical of Peak I and returned

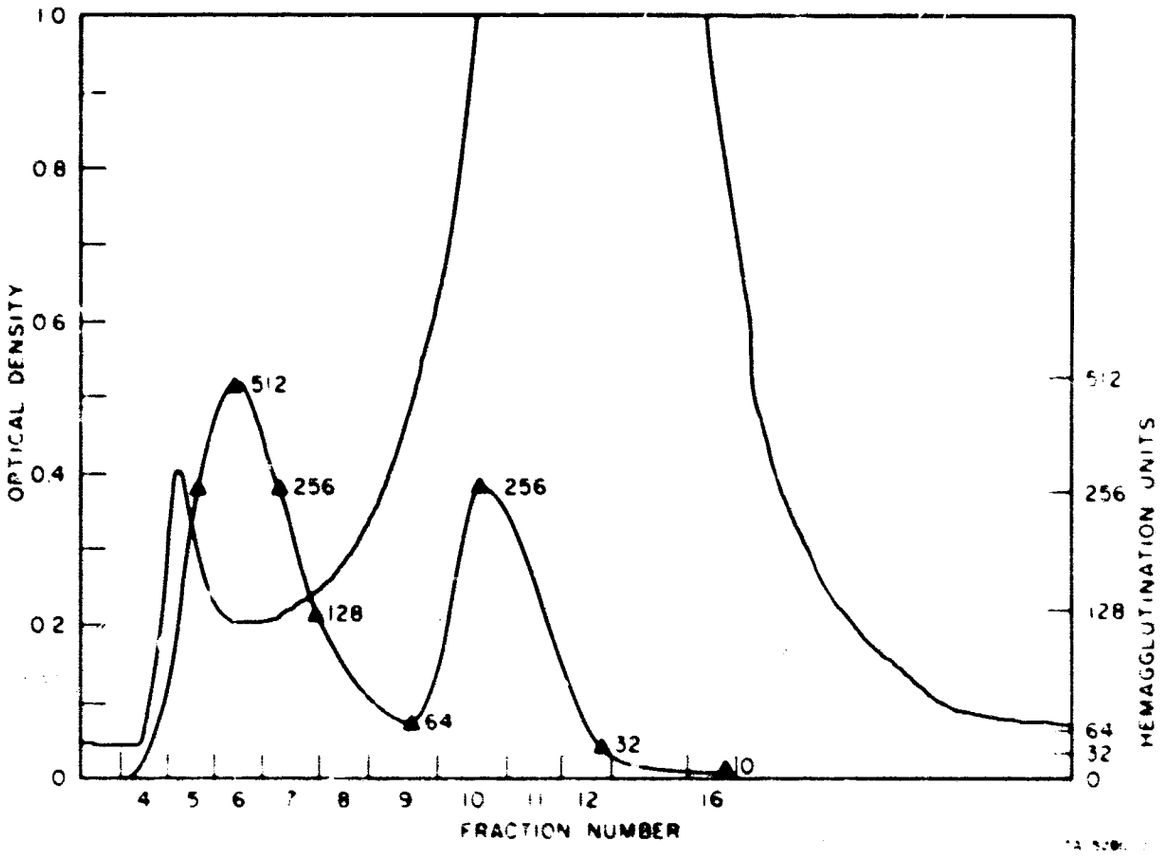

FIG. 1 ELUTION PATTERN OF PARTIALLY PURIFIED BOTULINUM TOXIN (Type A) THROUGH SEPHADEX G-200 IN HEMAGGLUTINATION BUFFER (pH 7.2)

to baseline until the absorbance rose on a large, broad peak typical of Peak II. The relative distribution of absorbance was 5% and 95%, respectively. Because Fraction 22 was well into the elution volume of the original Peak II, representing components that had to diffuse through the beaded gel matrix to emerge from the Sephadex column at the large retention volume, it is unlikely that the resultant Peak I material occurred by any mechanism other than aggregation.

Fractions 19 and 20, representing Peak II material, gave positive hemagglutination patterns to high dilutions, but both patterns began with a pro-zone phenomenon.

An attempt was made to investigate the hypothesis of dissociation-aggregation by chromatographing Fraction 10, the advanced part of Peak I of the original chromatogram. There was evidence only for Peak I, none for Peak II. This supports the conclusion that there is no measurable rate of dissociation at pH 7.2, at least to our limits of detectability.

An attempt was made to increase the resolution of components by using 4 g of Sephadex G-200 in the long 1.5-cm I.D. column. The crystalline toxin solution provided by Dr. Schantz was selected for chromatography. The toxin precipitated when sodium hydroxide was added to neutralize the solution. Not until the pH was raised above 9 did the solution clarify. Readjustment of the pH to 7.4 produced severe turbidity. Dilution with an equal volume of HA buffer aided solution, and the sample was loaded on the 4-g column with the hope that development with HA buffer would complete solution. Inexplicably, the turbid solution was virtually arrested at the top of the column, and the experiment was aborted.

Subsequent experiments were performed on a shorter 1.3-g column of Sephadex G-200 with dimensions of 1.5 cm x 26 cm. Figure 1 shows the optical density of the eluate. In addition, a portion of each 4-ml fraction was serially diluted and tested with chicken RBCs for HA activity. Fractions 4 through 10 gave dilution endpoints of 0, 256, 512, 256, 128, 64, and 256, consecutively. Fractions 12 and 16 were 32 and 0, respectively. Consequently, there were two peaks of HA activity. The first HA peak occurred in the more transparent region of the chromatogram between the two ultraviolet absorption peaks. We concluded that the HA activity was not related to the absorption at 280 mμ.

Amino acid analyses were performed on hydrolysates of combined fractions 4 and 5 and on fraction 7. The results are included in Table I (Cols. III and IV).

Experiments were performed in which stock toxin solution was absorbed by sheep and ox erythrocytes before chromatography under the usual conditions on the 1.3-g column of Sephadex G-200. During the absorption studies, there was a variable amount of hemolysis of the red cells. The pigmented hemoglobin, loaded on the column in the absorbed supernatant solution, eluted in effluent fractions near the volume equivalent to

total volume of the column. In comparison, the second peak of HA activity eluted before the hemoglobin. If the component in that peak is a monomeric form of the botulinum hemagglutinin, its behavior in gel filtration chromatography suggests a mol wt greater than 70,000. The results of these experiments suggest that there would be advantages to using the stroma fraction (elinin) for absorption-elution methods of purifying the hemagglutinin.

When a solution of crystalline urease (mol wt 480,000) was chromatographed under similar conditions to the toxin solution, the initial ultraviolet-absorbing peak appeared in the same location on the chromatogram as the first peak of HA activity. It should be apparent from Fig. 1 that fractions 5 through 8 contained most of the hemagglutinin loaded onto the column. These fractions were considered enriched in terms of HA units/weight of protein. However, the degree of toxicity in these fractions of the chromatogram remains to be determined.

Amino acid analyses were also performed on combined fractions of Peak I of toxin solution absorbed with ox cells (Column V, Table I).

We have attempted to draw some conclusions from the data in Table I. For example, when the composition of Peak I and Trough (both of which contain hemagglutinin) are compared with absorbed Peak I (containing no hemagglutinin), we conclude that the hemagglutinin-containing fractions have more of the following amino acids: aspartic acid, proline, glutamic acid, valine, isoleucine, tyrosine, and arginine. On the other hand, the hemagglutinin-containing fractions have less of the following amino acids: serine, glycine, alanine, lysine, and histidine. The hemagglutinin probably contains very little, if any, tryptophan because there is no relationship between ultraviolet absorption and HA activity.

The composition of the crystalline toxin from Dr. Schantz, as we analyzed it (Col. 6, Table I) had some unique features. The mole contribution of aspartic acid and glutamic acid considerably outweighs that of the basic amino acids, and explains in part the acidic isoelectric point of the toxin. There were significant proline and methionine contributions. Only a trace amount of cystine was observed. Histidine was the least abundant amino acid. Figure 2 is the pattern of amino acids in the acid hydrolysate of the crystalline toxin eluted under conditions for components in physiological fluids.

When the data for the amino acid composition, obtained in μmoles/ml sample, were calculated on a weight basis using "reduced" molecular weights of the amino acids in the conversion, some interesting results were obtained. The stock solution of crystalline toxin from Dr. Schantz contained 2.91 mg/ml against a reported 2.94 mg/ml. Thus, the recovery of amino acids, notwithstanding the undetermined tryptophan, was nearly complete. In addition, the total quantity of amino acids eluted in Peak I was 3.65% of the protein in the portion of stock toxin solution loaded on the Sephadex column. After absorption of the hemagglutinin by ox RBCs, the same fractions in the Sephadex chromatogram contained 2.97% of the original (unabsorbed) loading protein. The weight of hemagglutinin protein is a meager 0.68% of the partially purfied stock solution. Even if the toxin were five times as pure, it is not surprising that Wagman[23] concluded that absorption of the hemagglutinin did not change the amount of protein in a solution of the neurotoxin-hemagglutinin complex.

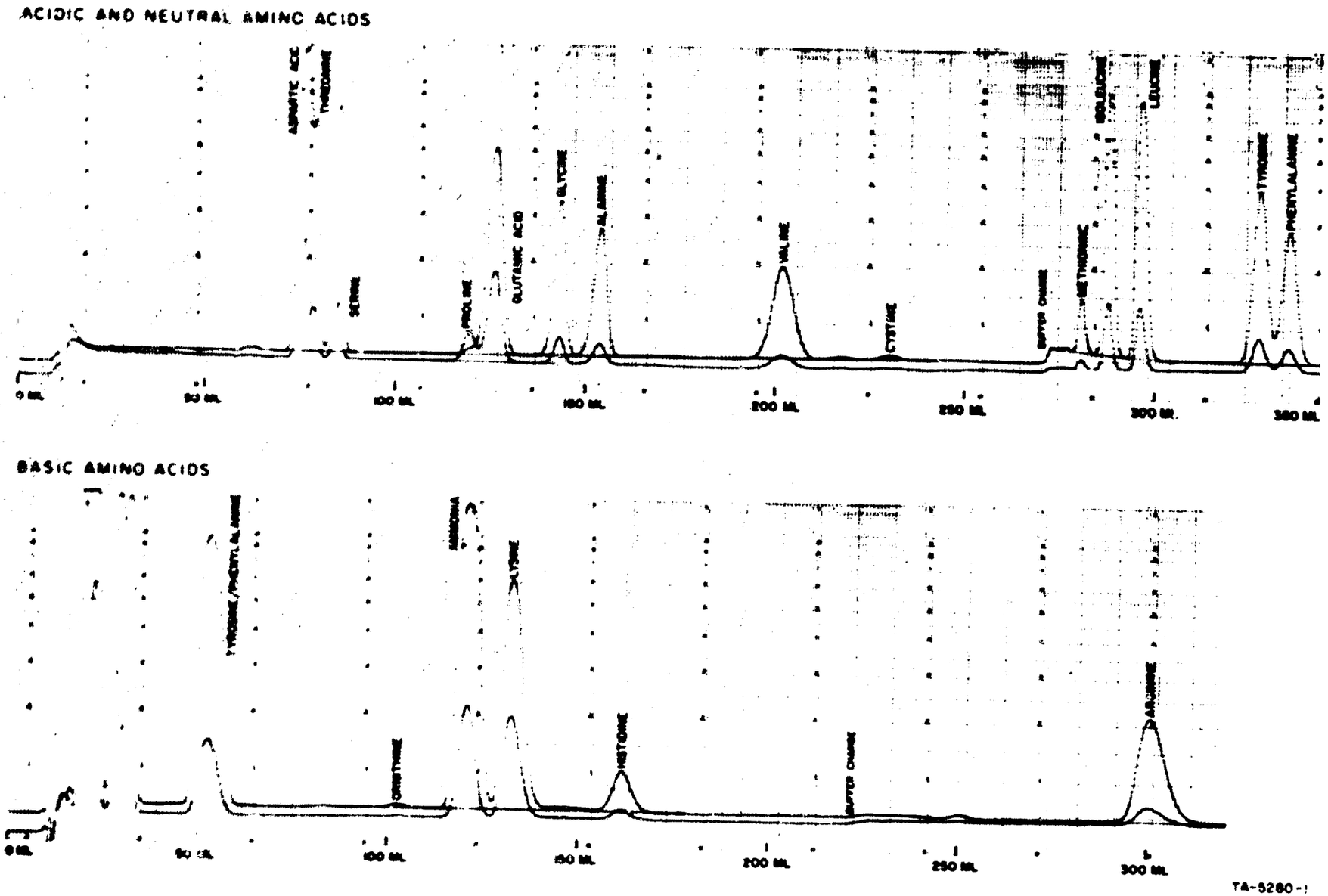

FIG. 2 ELUTION PATTERN OF AMINO ACIDS ON AN ACID HYDROLYSATE OF CRYSTALLINE BOTULINUM TOXIN (Type A) UNDER CONDITIONS FOR PHYSIOLOGICAL FLUIDS ON AN AUTOMATIC ANALYZER. Top tracing from 250 μg protein; bottom tracing from 500 μg protein.

Lowenthal and Lamanna[18] presented evidence of dissociation of neurotoxin and hemagglutinin which varied with pH, i.e., 18% at pH 6.5 to 86% at pH 8.0. It was based on the amount of neurotoxin absorbed with the hemagglutinin by chicken RBCs. Our results indicate that Peak I has undoubtedly a large mol wt (>200,000) and still possesses hemagglutination properties. However, it is possible that two substances with molecular weights between 200,000 and 900,000 could exist as components in Peak I. In fact, Meyer and Lamanna[20] stressed the possibility that at pH 4.38 the neurotoxin might have a molecular weight of about 190,000 and the hemagglutinin a molecular weight of about 4.5×10^6 based on diffusion rates. pH conditions were quite different from those used by Lowenthal and Lamanna[18] and toxicity has not been determined for this material. Thus, its further characterization is necessary.

Wagman and Bateman[23-25] concluded that the neurotoxin-hemagglutinin complex dissociates on the basic side of the isoelectric point (pH 5.5) into slowly sedimenting toxic components with molecular weight of about 40,000 to 100,000 and that this dissociation increases with ionic strength. We are in partial agreement with Wagman's conclusion that the agglutination titer appears generally to be a function of the degree of aggregation but not, however, with the toxin.

In the middle of June 1965, we discovered the paper on Sephadex G-200 chromatography of the type E precursor and trypsin-activated toxin by Sakaguchi, Sakaguchi, and Imai.[51] These workers used a 4-g column, but it was slightly wider in diameter and consequently shorter. Approximately an equivalent number of mouse units (58×10^6) were loaded, compared to our 40×10^6 MU. It is apparent that the position of the first peak, both precursor and trypsin-activated toxin, varied with the buffers used and with pH and ionic strength. In general, the type E components eluted at a column volume of 65 to 80 ml in comparison to our Peak I, which eluted at 35 ml (comparable to the excluded Blue Dextran 2000). They noted that the main peak of a preparation of urease (mol wt 480,000) was eluted in the same effluent volume as type E precursor or toxin.

In several of their elution patterns, especially those for the trypsin activated toxin, the chromatogram extended considerably beyond the total column volume. We observed the same effect (Fig. 1) with crude type A toxin solutions and attribute it to adsorption, but not ion exchange.

A similar chromatographic study of the separation of the neurotoxin and hemolysin of Cl. tetani by gel filtration on Sephadex G-100 was described by Hardegree.[52]

C. Agar Gel Diffusion Studies

Agar gel diffusion by the micro-Ouchterlony technique was tested on a solution of partially purified toxin as a method of qualitative analysis for the botulinum neurotoxin-hemagglutinin complex. Attempts to obtain precipitation bands between the toxoid solution and horse antisera were unsuccessful because the toxoid was too dilute. However, the toxin gave at least three lines of precipitation against this antiserum. The most intense line, presumably containing high molecular weight components, was much nearer the toxin well, and the two progressively less intense lines were adjacent to the major line but on the side toward the antiserum well. The heterogeneity and position of bands were not unexpected.

The Lederle horse antiserum is prepared with detoxified culture filtrate as the antigenic mixture. Consequently, antibody formation against a variety of components is likely. Since high purity toxin is reported to have a mol wt about 70,000, its diffusion in the 2% agar (buffered at pH 7.1) might be more rapid relative to the horse antitoxin antibodies, assumed to have mol wt 160,000. Smaller components in the neurotoxin-hemagglutinin mixture would diffuse more rapidly and precipitate between the line of the high molecular weight component and the antiserum well. Thus, in some respects, the agar diffusion pattern was similar to that described for native and photo-oxidized toxin of type A against rabbit serum by Boroff and DasGupta.[53]

The assignments of the precipitin lines are speculative, because of the lack of purity of the crude toxin.

More work must be done to determine the highest dilution of stock toxin solution (or, alternatively, the minimum number of mouse and HA units) that gives a detectable precipitin line. The micro-Ouchterlony technique might be useful for following changes in the components in peaks from Sephadex and other types of column chromatography. We estimate the toxin wells contained 50,000 MU/5 µl.

Another use for agar gel diffusion analysis would be to compare patterns before and after absorption of the hemagglutinin with RBCs. Lamanna[6] noted that addition of normal serum to a mixture of RBCs and toxin did not affect the HA titer. We interpret this result as indicating that normal serum components will not displace the hemagglutinin from agglutinated RBCs. However, Lamanna found that a mixture of sheep RBCs and horse antitoxin, incubated for 1 hr before the addition of toxin, remained unagglutinated; he concluded that the toxin and hemagglutinin were separate entities because toxicity was not absorbed by RBCs.

Even before the hemagglutination activity of botulinum toxin was discovered, Lamanna and Doak[15] reported that normal serum can be precipitated by toxin. Later, Lowenthal and Lamanna[18] demonstrated that some component(s) of normal horse or rabbit serum precipitated specifically

with the hemagglutinin and not with the toxin. Thus, one of the precipitin lines on our diffusion pattern (as well as that of Boroff and DasGupta[53]) may be "hemagglutinin-normal serum component" and the other a precipitin line of "toxin-antibody."

Schantz and Lauffer[54] studied the diffusion of substances through a column of agar. Schantz[55] observed that the toxin diffused much faster than the hemagglutinin. Further experimentation is required before the gel diffusion lines can be assigned with certainty.

D. Relationships between Hemagglutinins

A parallel interest in plant lectins (phytohemagglutinins) has proved useful for us in the study of the botulinum hemagglutinin. Bacto-Phytohemagglutinin-P, a protein lectin prepared commercially from red kidney beans,[56] is an example of this class of substance. A standard solution of PHA-P in HA buffer (15 mg/ml) had a dilution endpoint of 1:16,000 with chicken RBCs; the endpoint with horse RBCs was greater than $1:2 \times 10^6$, and was used as a substitute for the botulinum hemagglutinin in exploratory studies of HA determined in a Coulter Electronic Particle Counter.

A chicken cell suspension, diluted 1:100 from the usual concentration, gave counts of 32,000 in HA buffer under the experimental conditions. When the cells were suspended in a 1:2000 dilution of PHA-P solution, the total count gradually decreased. After 10 min it had decreased 13%; after 30 min, 68%. This study merits further investigation. Not only was it highly sensitive and rapid, but it should give more valuable information if the results are analyzed simultaneously by a distribution plotter. The instrument capable of performing these studies with the botulinum hemagglutinin was only recently made available to us.

Although only the one lectin is commercially available, much is known about the properties of a number of plant agglutinins. They show an unpredictable and broad range of variation in agglutination capacity with RBCs from different species. Most interesting to us are the molecular weights of these lectins, which vary as follows: soy bean, 96,000[57]; red kidney bean (PHA-P), 160,000[56]; lima bean, 54,000-80,000[58]; jack bean, 96,000[59]; castor bean, 77,000-85,000[60]; hempseed, 300,000[61]; and mushroom, 100,000.[62] Molecular weights as low as 54,000 lead us to assume that toxin as small as mol wt 70,000 could possibly have hemagglutinating capacity as a property of the same molecule. However, the low molecular weight toxins recently reported by the Dolman group[63] may not exhibit hemagglutination activity. We consider it very significant that the lectin of the jack bean, concanavalin A, can be detected by horse RBCs at a dilution endpoint of $1:10^7$.[64]

E. Comments on Low Molecular Weight Toxin

Dr. Carl Lamanna kindly brought to our attention the paper by Gerwing, Dolman, and Bains.[63] He suggested that it would be useful to

repeat the Canadian work and to determine whether or not the 12,000-mol wt material was hemagglutinin free. There were two logical starting materials: one was crystalline type A toxin, from which a low molecular weight material might be dissociated by control of pH and separated by Gerwing's chromatography conditions on DEAE-cellulose; the other was Dolman's "corn T" strain of Cl. botulinum type A, following Gerwing's procedure precisely.

A sample of lyophilized culture was requested and received from Prof. C. E. Dolman. Unfortunately, the experimental studies were not conducted, in part because we understood that Schantz and his colleagues were already studying this phenomenon of the low molecular weight toxin. Dr. Schantz informed us that attempts had been made to evaluate the molecular size of the toxin in bacterial cultures of U.S. strains of type A organisms. He found evidence substantiating high molecular weight toxin without exposure to allegedly detrimental conditions, such as low pH, organic solvents, or high salt concentrations, that might promote molecular aggregation.

After reading the Gerwing paper, some differences and anomalies were noted in the comparison of the new and old toxins and procedures. First, the "corn T" strain of type A organism was used; it produced toxin concentrations varying from 100,000 to 300,000 mouse MLD/ml. Our previous experience with toxin production was with a strain that consistently produced about 1×10^6 mouse MLD/ml. The toxin from these cultures has a high molecular weight. Gerwing and co-workers[63] also reported instability of the toxin above pH 7, in contrast to the observations of Spero[35] that the type A toxin is quite stable up to pH 10.

Secondly, the Canadian group used ammonium sulfate at 0.5-saturation for isolation of the crude toxin. This seems rather high in comparison with the values of 0.15 to 0.20 used by Lamanna et al.,[3] 0.3-saturation employed by Abrams et al.,[2] and 0.16-saturation used by Duff and his coworkers.[27]

The DEAE-cellulose chromatography was performed by the Dolman group at pH 5.6. Although this condition may still be in a useful pH range, it is a considerable departure from the ideal conditions stressed by Sober and co-workers,[65] especially in comparison with Fig. 4 in that paper. Dolman observed that the toxin was the first component to emerge from the DEAE-cellulose column; thus, it is the most basic protein in the mixture recovered by ammonium sulfate precipitation. In addition, the majority of the ultraviolet absorbing components all showed stronger affinity and were devoid of toxicity. The HA titration of components in the effluent from DEAE-cellulose columns should be most informative. In this regard, PHA-P was separated on DEAE-cellulose into three fractions of HA activity, by Prager and Speer.[66]

Finally, the use of 0.01 M citric acid (pH 2.2) is an extreme departure from the typical treatment of toxin solutions. Lindner, Elmqvist, and Porath[67] demonstrated that the relatively basic, low molecular weight polypeptides oxytoxin and vasopressin were dissociated from

complexes with typical proteins under acid conditions (1 N formic acid). They then applied gel filtration through a column of Sephadex G-25 to separate the components according to molecular size.

There is another criterion of minimum molecular weight, namely, amino acid composition. Some work in this area has been performed on type E toxin by Gerwing et al.,[68,39] using histidine as the limiting amino acid. In the case of type A toxin, we must somehow reconcile the amino acid compositions of the fundamental toxin unit and the macromolecular aggregate with mol wt 900,000.

VI RECOMMENDATIONS

As a consequence of this research, the following topics are recommended for further investigation:

1. The preliminary successful separation of most of the type A botulinum hemagglutinin within a moderately high molecular weight fraction by Sephadex chromatography under neutral pH conditions suggests pursuing this approach to the concentration and isolation of purified hemagglutinin. It would be preferable to perform research on samples of the neurotoxin-hemagglutinin complex in the highest state of purity (toxicity).

2. The Sephadex G-200 elution pattern should be investigated for toxic activity. The toxic nature of the high molecular weight components under neutral conditions should be thoroughly studied. Gel filtration chromatography should be performed at higher pH and in dilute acetic acid solution.

3. Absorption-elution methods should be used to isolate the hemagglutinin on the elinin fraction of ox erythrocyte stroma to avoid problems of hemolysis and contamination.

4. Parallel investigations on neutral solutions should be conducted by the technique of density gradient centrifugation.

5. Alternative methods of protein analysis, such as Disc electrophoresis, immunoelectrophoresis, and immunodiffusion, should be examined as criteria of purity and homogeneity to complement amino acid analyses as the principal criterion.

6. The preparation of low molecular weight toxin under the conditions of Gerwing, Dolman, and Bains[63] should be repeated and confirmed and examined for the occurrence of hemagglutination activity.

7. Electronic particle counting methods should be investigated as a method of improving the sensitivity and discrimination of detection for botulinum toxin.

ACKNOWLEDGMENTS

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Details of the work described in this report are recorded in Stanford Research Institute Notebooks 6839, 6841, and 7975.

Lloyd M. Moss, Ph.D.
Biochemist

LKM:jg/bm eh

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Menlo Park
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Toronto, Ontario, Canada
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67 Yonge Street Room 710
Toronto 1, Ontario Canada

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Milan, Italy

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